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Comparison between eDNA and traditional morphological methods for fish diversity monitoring in rivers

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Environmental DNA (eDNA) is increasingly employed for biodiversity monitoring, yet its accuracy and consistency relative to conventional fish surveys remain under debate. Here, we evaluated fish assemblages at 14 sites across six major rivers in Liaoning Province, China, using four primer sets (12 S rRNA, COI, MiFish, and a high-variation MiFish) coupled with high-throughput sequencing, and compared results with capture-based surveys. eDNA detected 211 species across 17 orders, 71 families, and 146 genera—substantially exceeding traditional methods—but failed to identify nine species recorded morphologically and could not provide ecological attributes such as body size or population structure. Species richness and composition varied between tributary and mainstem sites, reflecting hydrological and habitat gradients. Detection efficiency also differed among primers, underscoring the importance of multi-primer strategies. Overall, eDNA provides a sensitive and efficient complement to conventional surveys, but its limitations in taxonomic resolution and ecological inference highlight the need for integration of molecular and conventional morphological methods to achieve comprehensive fish diversity assessments.

Keywords Freshwater fishes, Species richness, Environmental DNA, Conventional sampling, Complementary approaches

Freshwater biodiversity is experiencing steep declines due to pollution, habitat alteration, overexploitation and biological invasions[1,2]. As central components of riverine ecosystems, freshwater fishes contribute to nutrient cycling and food-web dynamics and are widely used as indicators of ecosystem condition[3]. Yet monitoring these changes remains challenging: conventional surveys (e.g., gill-netting, electrofishing, visual identification) yield direct biological information but are invasive, labour-intensive, costly and reliant on taxonomic expertise. These constraints motivate complementary, low-impact approaches[4].

eDNA metabarcoding offers such a complement[5]. First used to detect invasive American bullfrogs (Rana catesbeiana) in fresh waters[6], eDNA has since been widely applied to survey aquatic biodiversity across rivers, lakes and marine systems[7,8]. In China, eDNA applications are rapidly expanding, including riverine assessments in Beijing (Chaobai and Beiyun Rivers) and the Danjiang River, as well as river–lake networks and estuaries (Pearl River)[9], collectively demonstrating feasibility across diverse hydrological settings[10,11].

Compared with traditional surveys, eDNA can detect species without capture, reduce ecological disturbance and often improve sensitivity for rare or elusive taxa[12,13]. However, performance depends on primer choice, environmental conditions and reference-database completeness[14], and false positives can arise from DNA persistence or contamination[15]. Thus, strengths and weaknesses must be weighed in context, and eDNA is best viewed as a valuable but non-standalone tool for comprehensive monitoring[16].

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Here we compare eDNA metabarcoding with conventional surveys across six rivers in Liaoning Province, China. We targeted two mitochondrial markers—12S rRNA and cytochrome c oxidase subunit I (COI)—and used widely adopted primer sets for 12S rRNA (MiFish-U and a high-variation MiFish set) and validated mini-barcode primers for COI, then contrasted these results with cage-fishing data to evaluate detection sensitivity and taxonomic coverage. By analysing concordance and divergence between approaches, we propose an integrated framework that leverages the complementarity of molecular and traditional methods to optimize fish biodiversity monitoring in riverine ecosystems.

Materials and methods

Study area and sampling locations

Based on hydrography, channel morphology, and confluence structure of the Liao River Basin[17], we established 14 riverine sampling sites in September 2024 (autumn). Sites were distributed as follows: Xiaoling River (A1–A3), Liao River mainstem (A4–A6, A8, A9, A11), Dayang River (A7), Qing River mouth (A10), Taizi River (A12–A13), and Yingna River (A14). The spatial arrangement captured longitudinal and tributary gradients as well as the estuarine transition (Fig. 1).

Sample collection and processing

eDNA water sampling and laboratory processing

At each site, 15 L of surface water (0–30 cm) was collected using a sterile water sampler and kept on ice (~ 4 °C) during transport. Before each sampling event, all reusable equipment was disinfected with 10% sodium hypochlorite and rinsed thoroughly with deionized water; single-use consumables were employed whenever possible to minimize contamination risk[18]. In the laboratory, water from the same site was composited and split into three technical replicates (4 L each); the remaining 3 L was discarded following common practice[19],Luo et al., 2015; Xu et al., 2016). Replicates were filtered within 24 h through 0.45 µm mixed-cellulose-ester membranes (Whatman, UK) using a vacuum manifold. When turbidity or suspended sediment was high, samples were pre-filtered in the field through sterile medical gauze[20,21]. Filtration units were decontaminated between samples as above. We processed field/lab blanks (2 L molecular-grade water) alongside samples to monitor exogenous DNA. Filters were stored at − 80 °C until extraction.

Total DNA was extracted from filters using the PowerWater DNA Isolation Kit (Qiagen Genomic-Tips) per the manufacturer's protocol. DNA quality was screened by 1% agarose gel electrophoresis[22,23]. Each replicate was extracted independently, and unused filters served as extraction blanks. DNA was stored at − 20 °C prior to PCR.

Conventional capture-based sampling and specimen processing

To generate a morphology-based benchmark, we conducted standardized capture-based surveys at the same site. At each site we deployed one 6 m ground cage (0.5 m × 0.5 m; 1.5 cm mesh) for a 24 h soak (start: late afternoon; retrieval: next day), positioned along near-bank habitat with reduced current. This effort (net length and soak

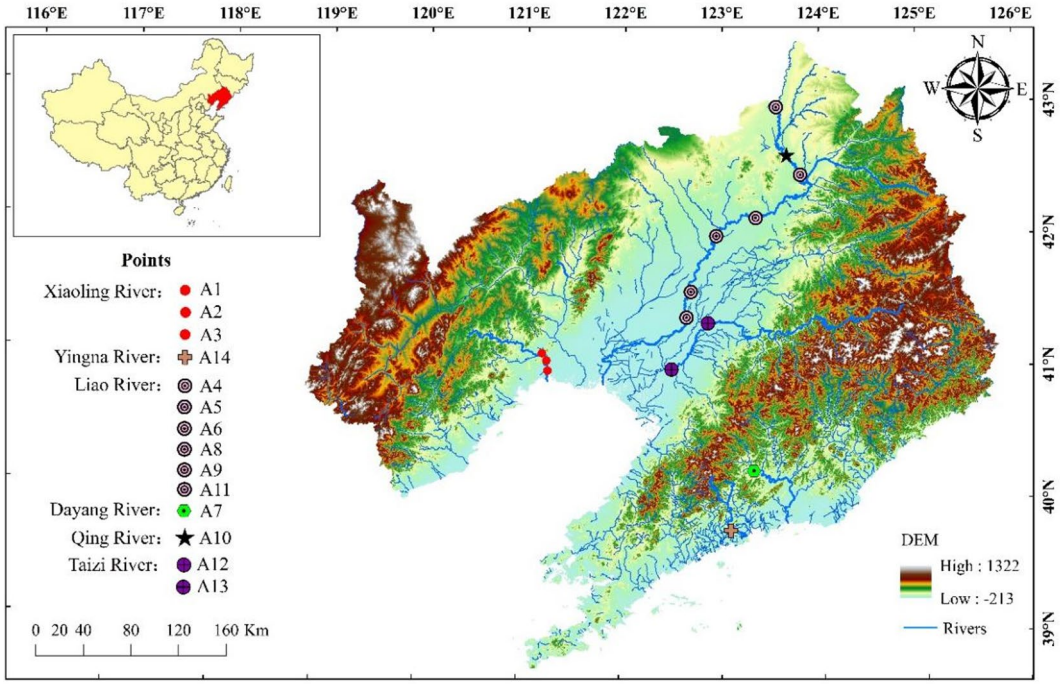

Fig. 1. Spatial distribution map of the study area in the Liao River Basin and location map of sampling sites, where A1, A2 and A3 are Xiaoling River, A4, A5, A6, A8, A9 and A11 are Liao River, A7 is Dayang River, A10 is Qing River, A12 and A13 are Taizi River, A14 is Yingna River.

time) was standardized across sites. Where hydrodynamic conditions permitted, we additionally used set/hang nets or dip (plunge) nets to supplement species detection; ancillary catches were recorded as presence-only and were not used in quantitative comparisons. Specimens were identified in situ whenever possible; individuals requiring lab confirmation were preserved on ice or in 5–10% neutral buffered formalin to prevent tissue degradation.

Taxonomic identification

eDNA technical identification

To increase coverage of fish diversity, we targeted two mitochondrial markers—12S rRNA and cytochrome c oxidase subunit I (COI)—with validated primer sets. For 12S rRNA, we used the MiFish-U primers and a high-variation MiFish set; we also included a generic fish 12S rRNA primer pair to cross-validate detections. For COI, we used a commonly adopted fish mini-barcode primer pair. PCRs (25 μL) contained 12.5 μL 2 × Taq Master Mix, 1 μL each primer (10 μM), 2 μL DNA template, and 8.5–9.5 μL nuclease-free water; cycling followed primer-specific recommendations. Each extract was amplified in triplicate PCRs with PCR negatives included. Amplicons were visualized by agarose gel, pooled per replicate, gel-purified, and libraries were prepared with Illumina TruSeq kits. Sequencing used MiSeq v3 2 × 300 bp paired-end chemistry. The details referto·Figure. S1.and Table. S1.

Morphology-based identification of captured fish

Fish were identified to the lowest possible taxon using standard keys(Sofia D. et al., 2025); when necessary, vouchers were confirmed in the laboratory by detailed morphological examination (meristics, morphometrics) with body length and mass recorded. Diet (stomach contents), gonadal development, and age structure (as feasible) were assessed to obtain ecological information. Taxonomy followed FishBase (https://www.fishbase.de/) and Species and Distribution of Inland Fishes of China[24].

Bioinformatics and statistical analyses

Raw sequences obtained from high-throughput sequencing were subjected to quality control, filtering, splicing, and clustering to generate species abundance data. Operational taxonomic units (OTUs) were clustered at a similarity threshold of ≥ 97%[25] Representative OTU sequences were annotated using the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/) and MitoFish (http://mitofish.aori.u-tokyo.ac.jp/) databases(version 4.09, accessed on 8 February 2025). To ensure accuracy, taxonomic assignments were cross-validated against distribution records of aquatic organisms in typical watersheds of Liaoning, and non-fish sequences were manually excluded. Based on the OTU clustering results, fish species composition and α-diversity indices were calculated. Statistical analyses and data visualizations, including Venn diagrams, bar plots, and heatmaps, were performed using R (v.4.4.1)[26,27] with the packages ggplot2[28] and vegan[29].

Results

Identification of fish species composition based on eDNA technology

Using eDNA to analyse water samples from typical rivers in Liaoning Province, we detected 211 fish species spanning 17 orders, 71 families, and 146 genera, with Cyprinidae contributing the largest number of taxa. As shown in Fig. 2, primers (a) and (c) recovered more taxa—particularly at the genus and species levels—than primers (b) and (d), indicating broader coverage and higher resolution at lower taxonomic ranks. By contrast, primers (b) and (d) yielded fewer assignments overall, consistent with narrower coverage and potential taxonomic bias.

Because the four eDNA primer sets are variants of the same molecular approach, primer-wise contrasts are treated as within-eDNA performance comparisons. Any comparison with the capture-based morphological survey is made at the method level, using the pooled eDNA species list (rather than primer-by-primer results), to acknowledge the non-equivalence of four eDNA assays versus a single morphology-based method.Fig. 3 presents the ten dominant fish genera per site, with co-occurring genera shown side by side, totaling 26 genera. Specifically included:*Schizothorax*、 *Acheilognathus*、 *Rhodeus*、 *Carassius*、 *Channa*、 *Neosalanx*、 *Rhinogobius*、 *Glossogobius*、 *Silurus*、 *Hypophthalmichthys*、 *Triplophysa*、 *Onychostoma*、 *Odontobutis*、 *Tor*、 *Mugil*、 *Cyprinus*、 *Macropodus*、 *Hypomesus*、 *Oreochromis*、 *Microphysogobio*、 *Rhinogobio*、 *Chanodichthys*、 *Kuhlia*、 *Megalobrama*、 *Larimichthys*、 *Acipenser*、 *Opsariichthys*。Of these, the genera *Channa* and *Hypophthalmichthys* were present in all sites, demonstrating their wide distribution in the region (genera tied for the top ten occurrences have also been included).

Fish biodiversity

Fish alpha diversity

Figure 4 presents rarefaction curves for the four primer sets (A–D), with sequencing depth on the x-axis and the number of observed features on the y-axis. In all cases, the number of features increased with sequencing depth and approached saturation at higher read numbers. Primer C tended to reach saturation between 60,000 and 80,000 reads, suggesting relatively higher efficiency, whereas primers A and B required 80,000–100,000 reads before leveling off. Primer D consistently produced fewer observed features than the other sets, with curves stabilizing earlier and at lower values. These patterns indicate variation among primers in their ability to recover community diversity, with primer C yielding comparatively higher richness and primer D showing lower sensitivity.

Figure 5 shows rank–abundance distributions of OTUs for the four primer sets (A–D). All curves exhibited long-tailed patterns, with a few high-abundance OTUs and many rare OTUs. Primers B and C produced broader curves with more OTUs and a more even distribution of abundant and rare taxa, whereas primers A and D

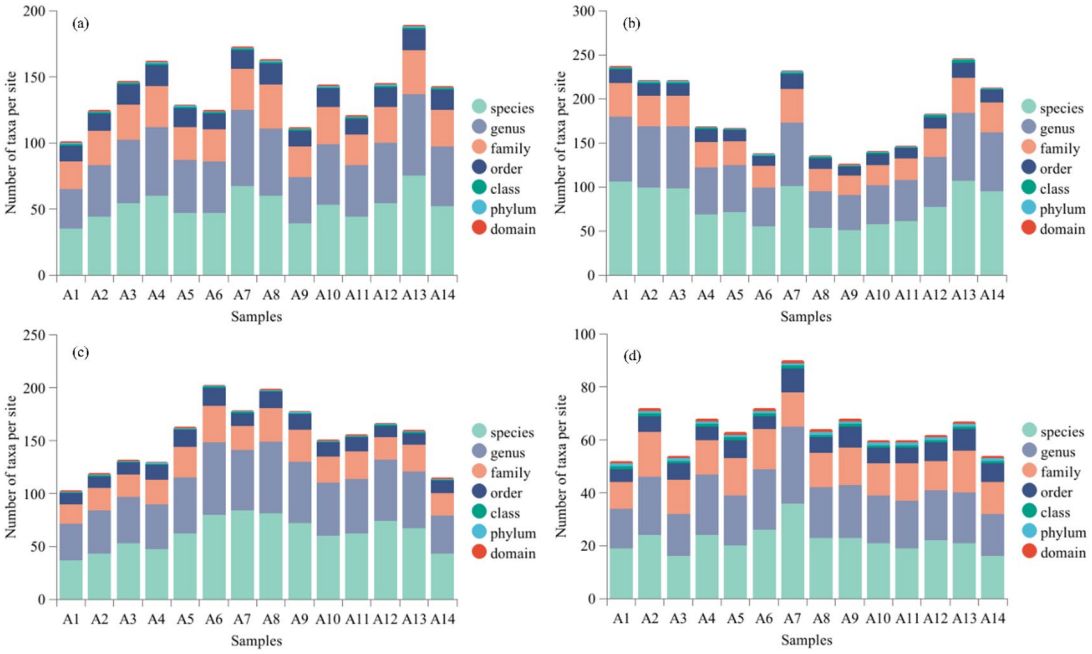

Fig. 2. Statistics on the results of OTU delineation and classification status identification in typical watersheds of the Liao River. *Note*: Figure (**a**)-Figure (**b**) Results of four primers identified by fish mitochondrial 12S rRNA gene amplification, COI, fish mitochondrial 12S rRNA amplification Mifish, and fish mitochondrial 12S rRNA amplification Mifish**, respectively.

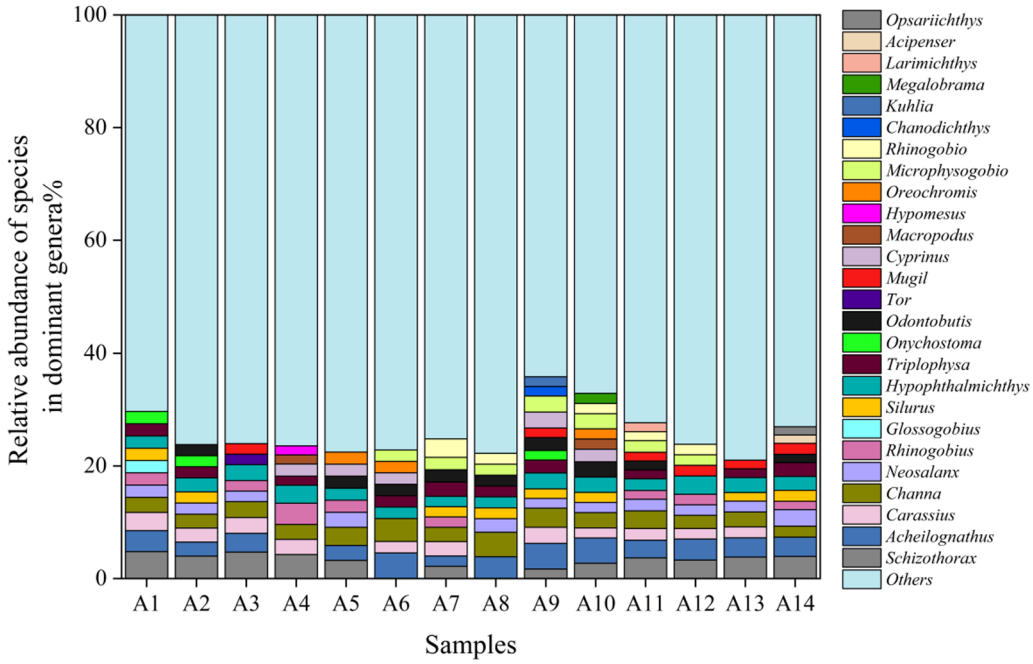

Fig. 3. Relative Abundance of Fish Dominant Genus Species Between Sampling Sites. Note: The top ten dominant fish genera were selected for this paper, and genera occurring side-by-side were included in the analysis to fully characterize the fish community in the region.

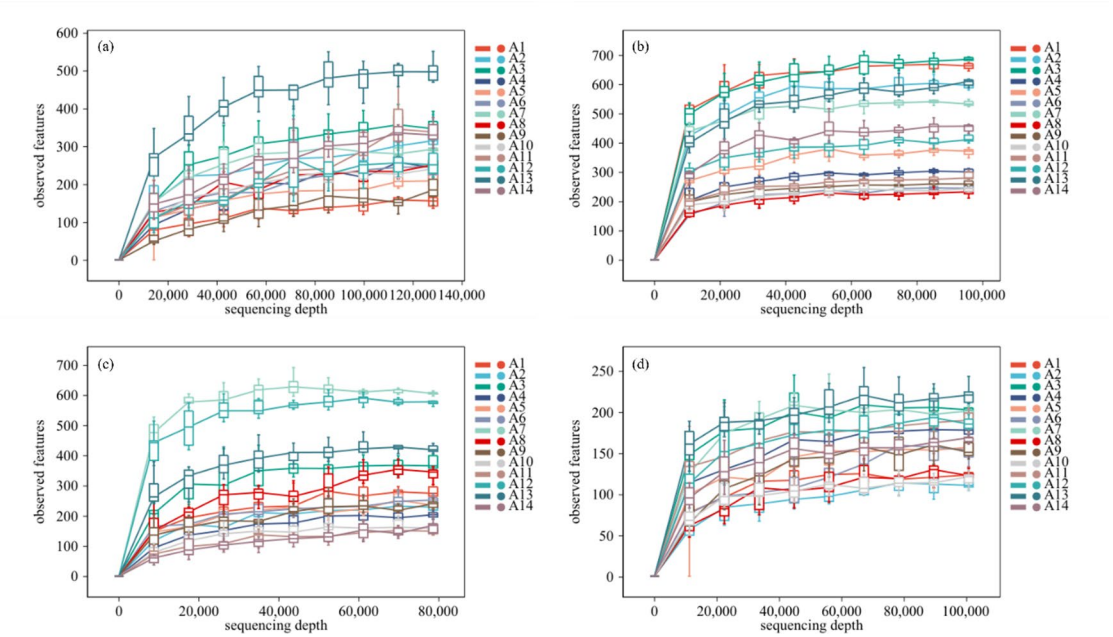

Fig. 4. Species alpha diversity index sparse curves for 4 primers.

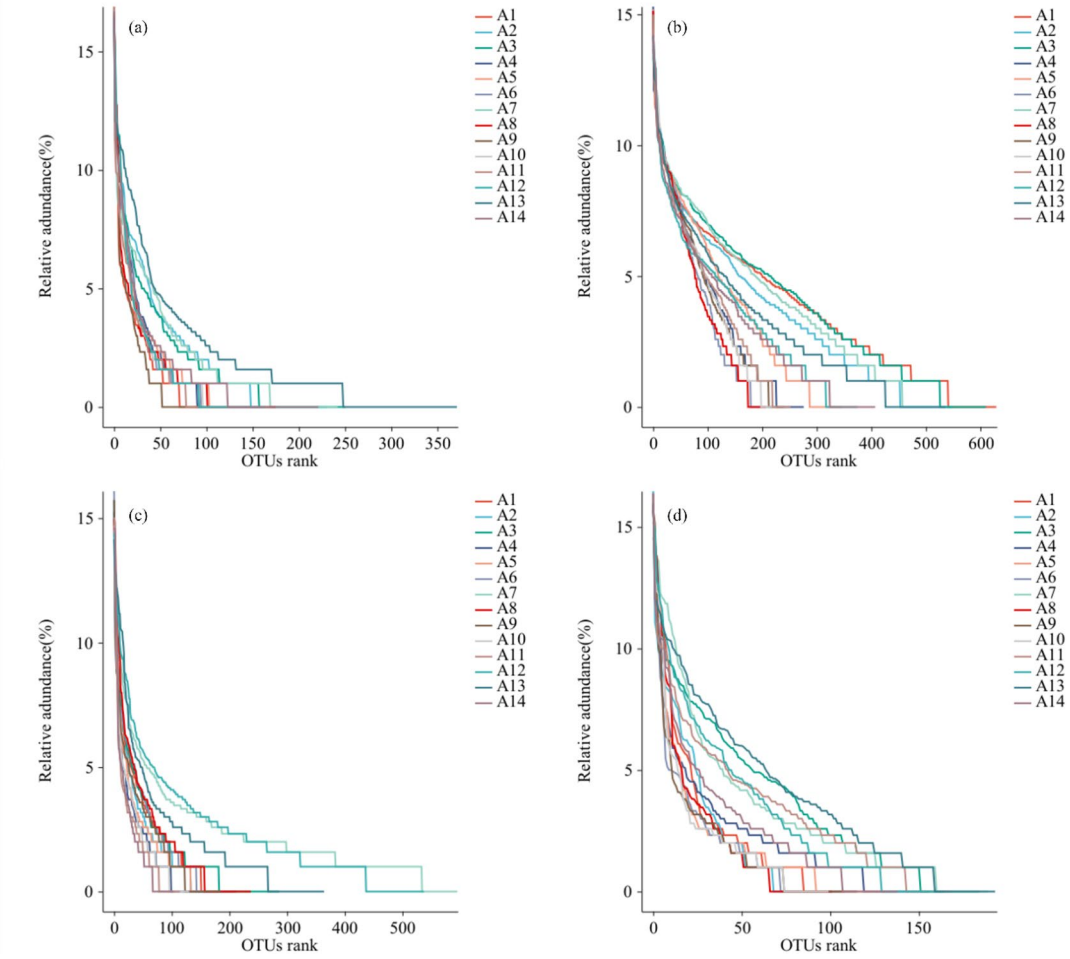

Fig. 5. Clustering curves of species alpha diversity index ranks for the four primers.

showed narrower curves with fewer OTUs and steeper declines in relative abundance. Differences in abundance patterns were evident among primers and across samples.

Beta biodiversity of fish

Figure 6 shows principal coordinate analysis (PCoA) of fish communities across 14 sites using four primer sets (A–D). The proportion of variance explained by PCo1 and PCo2 differed among primers, ranging from 29.1% + 18.7% for primer C to 65.8% + 11.0% for primer A. Sample distributions also varied: primer A produced more dispersed patterns, indicating greater community differentiation, whereas primers B and C showed clearer grouping of sites. Primer D accounted for 38.3% + 25.5% of the variance and revealed distinct inter-cluster separation. Together, these results indicate that community structure patterns depend on primer choice.

Figure 7 shows hierarchical clustering of fish communities based on the four primer sets (A–D), using the ten most abundant genera. Each subfigure combines a dendrogram with bar plots of relative abundance. Primer A produced clusters dominated by a few genera, whereas primer B showed more even distributions. Primer C yielded balanced clustering with smaller differences among groups, while primer D formed tighter clusters with greater dominance by a few taxa. Overall, clustering patterns differed among primers, reflecting variation in community structure and dominance of key genera.

Analysis of fish species differences between sites under four different primers

Figure 8 illustrates the numbers of shared and unique OTUs across 14 sampling sites for the four primer sets (A–D). The number of shared OTUs ranged from 7 (primer D) to 14 (primer B), while the number of unique OTUs varied widely among sites and primers. Site A7 consistently showed the highest number of endemic OTUs across all primers, reaching over 300 for primers B and C, whereas some sites (e.g., A10) yielded few or none. Overall, primer B detected the greatest number of shared OTUs, indicating higher overlap among sites, while primers B and C also revealed strong species uniqueness. Primer D produced the lowest values for both shared and unique OTUs, reflecting weaker detection capacity.

Figure 9 shows heatmaps of species composition across 14 sites under the four primer sets (A–D). Abundance patterns varied markedly among sites and primers. Under primer A, certain species were enriched at sites A2 and A3 but scarce elsewhere. Primer B detected species such as *Leuciscus idus* and *Neosalanx taihuensis* with strong geographic variation. Primer C revealed higher diversity at sites A7 and A12, with taxa such as *Mugil cephalus* and *Acipenser sinensis* concentrated at these locations. Primer D highlighted species enrichment at site A7, while distributions at other sites were more scattered. Overall, the primers differed in their detection of species abundance and clustering patterns, reflecting primer-specific sensitivity to community composition.

Comparison of eDNA technology with conventional morphological methods

A total of 19 fish species from 4 orders, 9 families, and 19 genera were captured and identified across the 14 sites using capture-based morphological surveys. The catch was dominated by Cyprinidae (73%), followed by

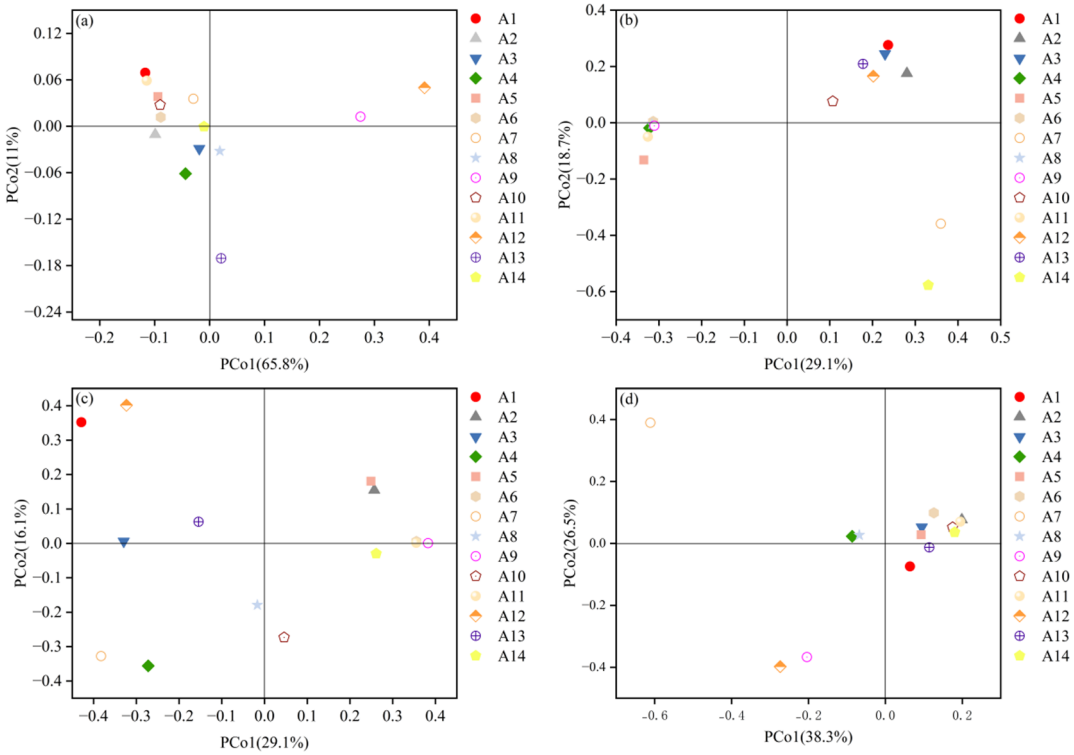

Fig. 6. PCoA principal component analysis of the principal coordinates of each point under 4 primer assays.

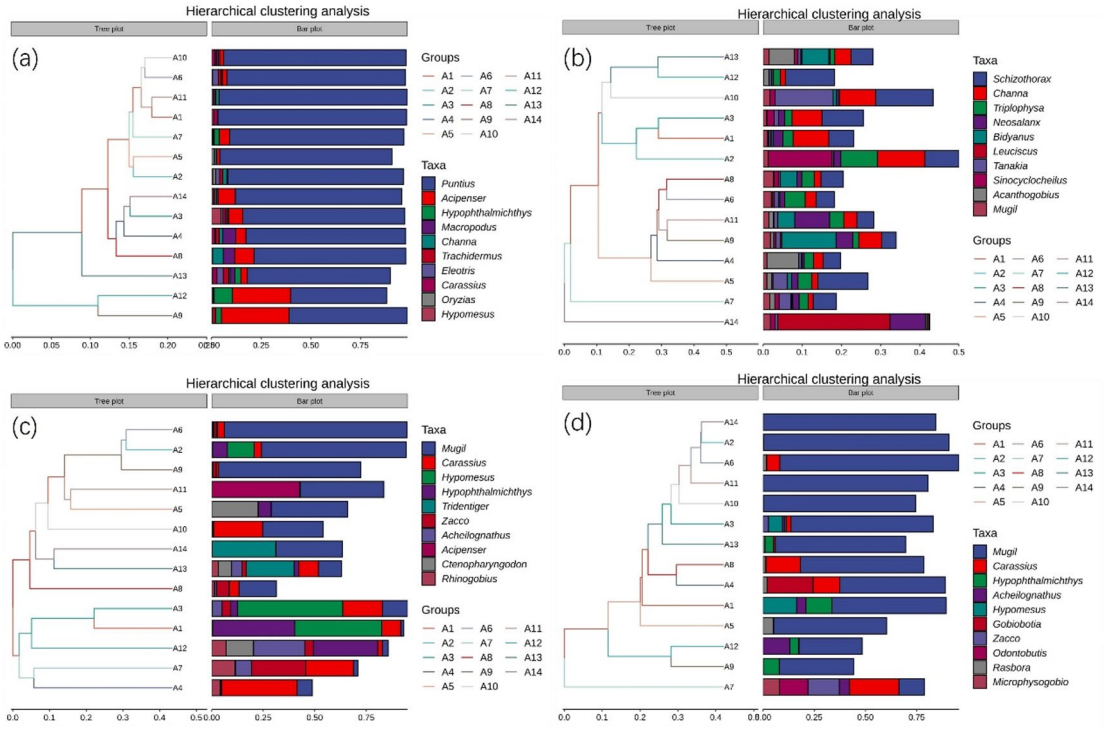

Fig. 7. Hierarchical clustering analysis of species β-diversity indices for 4 primers.

Cobitidae (8.89%), with a third family contributing 5.56%. The most abundant species were white minnow (n = 16) and common carp (n = 10). Species recorded by capture but not recovered by eDNA included *Rhynchocypris lagowskii*、 *Hypophthalmichthys molitrixa*、 *Sea catfish*、 *Pelteobagrus fulvidraco*、 *Hypseleotris swinhonis*、 *Perccottus gleni*、 *Cobitis taeniaLinnaeus*、 *Odontobutis obscurus*、 *Lophius litulon*. representing ~ 4% of the overall species pool. In comparison, eDNA identified a total of 211 fish species across the basin (Fig. 10).

Table S2 indicates that nine species recorded using conventional morphological methods surveys were absent from the eDNA results.

Discussions

Species composition of fishes in typical watersheds of Liaoning based on eDNA technology

This basin-wide eDNA survey represents the first multi-marker assessment of fish diversity in the Liao River[30], and it yielded a region-wide total of 211 species spanning 17 orders, 71 families, and 146 genera—demonstrating the power of eDNA to generate broad biodiversity inventories and to resolve among-site differences in community composition[31]. *Cyprinidae* dominated species richness and relative abundance, aligning with[32] and likely driven by the family's adaptation to fast, cold, montane rivers—conditions that promote widespread occurrence and high local abundance in our samples.The multi-primer strategy (two 12S assays plus COI) revealed clear performance differences, aligning with evidence that MiFish markers generally provide superior species-level resolution in freshwater systems[33]. Importantly, primer detectability is moderated by eDNA degradation and transport, driven by flow velocity, turbidity, temperature, and microbial activity[34]. Combining markers therefore maximizes taxonomic recovery and minimizes locus-specific biases[35,36]

Marked spatial heterogeneity in species richness reflected watershed-scale gradients. Tributaries (A1–A3, A7, A14) supported higher local richness of narrow-niche taxa, whereas mainstem sites hosted broader-tolerance assemblages. Restricted detection of Mugil cephalus and Acipenser sinensis at A7 and A12 further highlights how hydrology, resource availability, and human impacts interact to shape local communities[37]. These patterns suggest that hydrological connectivity and habitat filtering act as the predominant ecological processes shaping α-diversity, a conclusion that aligns with previous empirical evidence reported by Valentini et al. and Olds et al.[38],Olds et al., 2009).

Analysis of fish diversity in typical watersheds of the Liao River based on eDNA technology

Substantial primer effects were evident in both α- and β-diversity metrics. Primers B and C achieved higher richness saturation and more even rank–abundance distributions than primers A and D, emphasizing the significance of marker choice in eDNA-based community profiling[39–41].

Primer A explained the highest PCoA variance but generated greater among-site dispersion, whereas primers B and C produced clearer, ecologically interpretable clustering. These findings mirror earlier reports that 12S rRNA markers reliably resolve freshwater β-diversity patterns[42,43]. Despite methodological variation, all primers captured consistent basin structure: tributaries formed distinct clusters from mainstem sites, and Taizi River

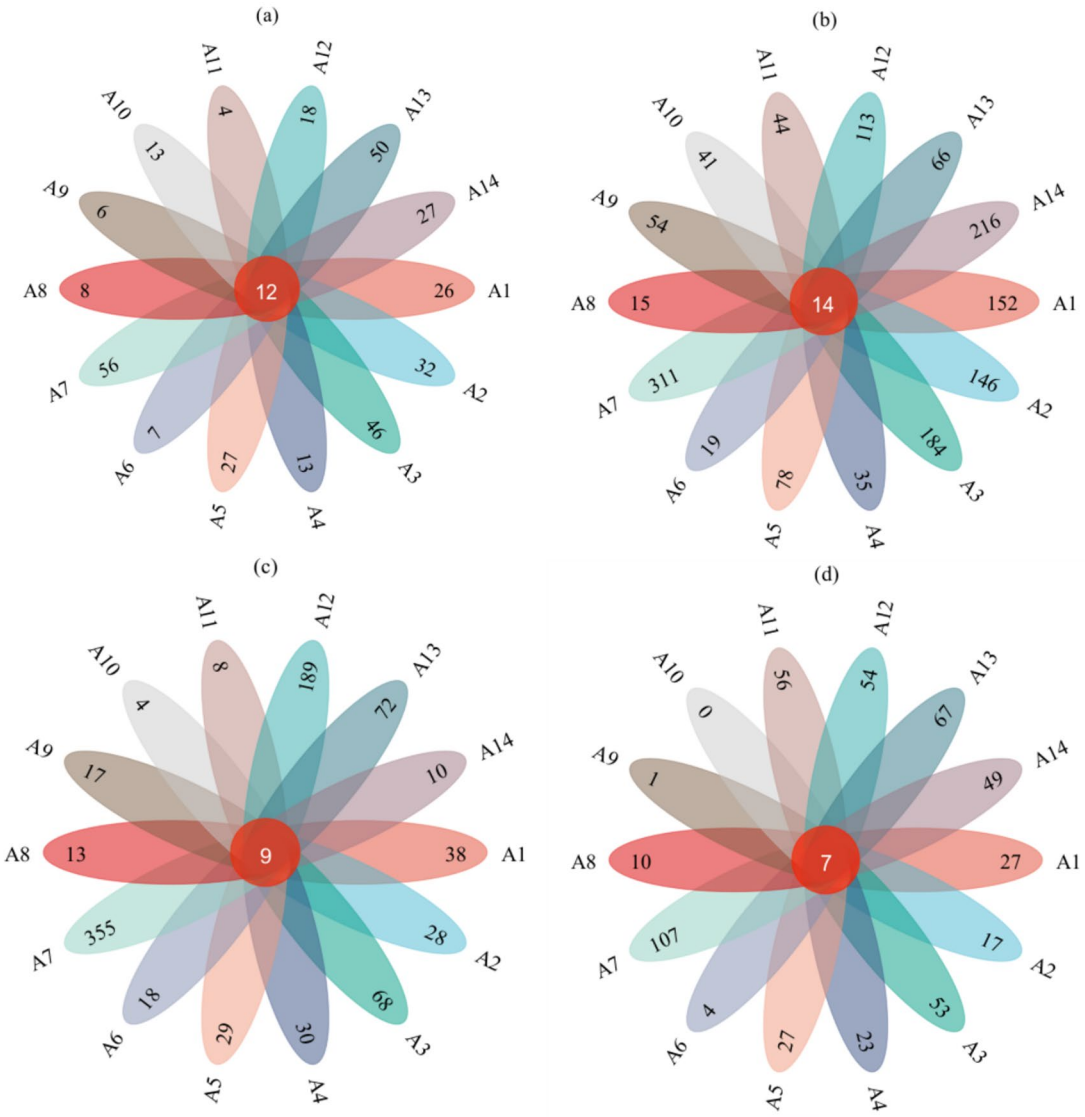

Fig. 8. Petal map of common OUT at different sampling points under 4 primers.

samples grouped tightly, reflecting shared species pools and hydrological regimes. Thus, ecological signals underpinning community turnover remain robust to primer choice.

Exploring the advantages and disadvantages of eDNA technology over conventional morphological methods methods

eDNA is a powerful tool for ecological research and aquatic biodiversity monitoring, offering several advantages over traditional capture-based surveys[44,45]. It does not rely on on-site taxonomic expertise, thereby reducing observer bias; it is highly sensitive, allowing detection of elusive or rare species; and it enables efficient processing of large sample volumes, saving time and labor[46]. In the study, eDNA detected 211 species across the Liao River, substantially more than the traditional method. However, capture-based surveys still identified nine species not recovered by eDNA, highlighting the complementarity of the two approaches[47,48]. Some discrepancies may be explained by exogenous or "legacy" DNA inputs from upstream transport, aquaculture effluents, or predator feces and carcasses, which can decouple DNA presence from live local populations.

Despite its promise, eDNA currently faces several limitations. False positives primarily stem from sample contamination, laboratory cross-contamination, or bioinformatic errors that misassign sequences(Fonseca, V.G. et al.,2023). Conversely, false negatives frequently occur due to the absence of target taxa from reference databases(Kirtane et al., 2021), preventing species assignment, or because of low eDNA concentration in the environment that falls below the detection limit. These limitations underscore the necessity of stringent laboratory controls and robust bioinformatic filtering(Martin P. et al.,2025). Closely related species remain difficult to discriminate due to high sequence similarity, and eDNA cannot provide critical biological information such as body size, age, sex, biomass, or reproductive status[49,50]. Moreover, mitochondrial inheritance complicates the

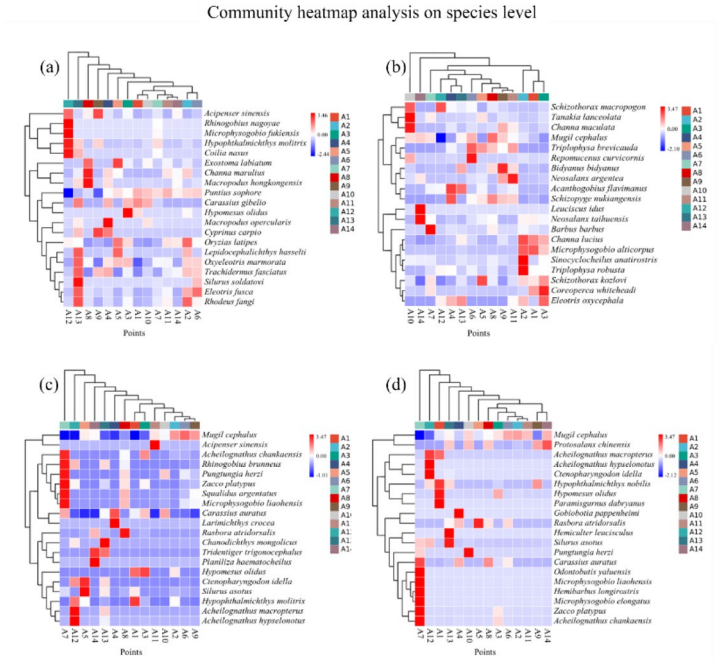

Fig. 9. Heat map of species composition at different sampling sites under 4 primers.

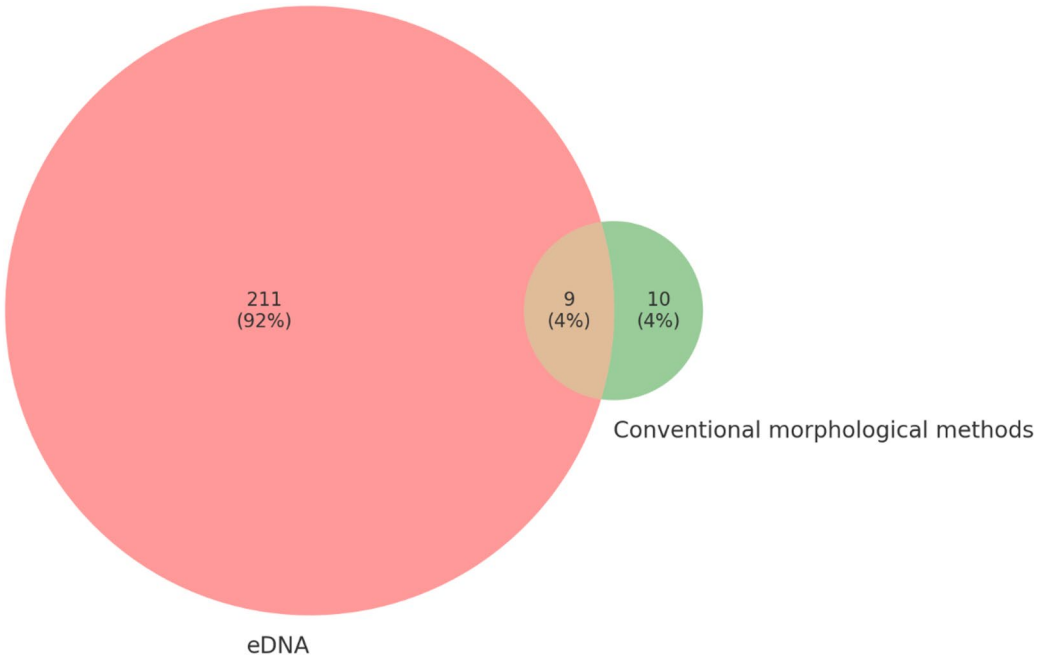

Fig. 10. Wayne's diagram of eDNA methods versus conventional morphological methods methods for investigating species types. *Note*: The middle section indicates species identified by traditional methods but not by eDNA methods.

detection of hybrids[51]. High-throughput sequencing itself can introduce erroneous sequences differing from true biological sequences by a few bases[52–54].

In summary, while eDNA greatly expands species detection, it cannot yet replace capture-based surveys. Instead, the two methods are best viewed as complementary: eDNA provides broad, non-invasive biodiversity screening, while traditional methods deliver essential ecological and demographic data. This complementarity offers practical value for biodiversity monitoring, supporting more robust river health assessments and improving the detection of rare or protected species(Kopp et al., 2023). Continued optimization of primer

design, reference databases, and analytical pipelines will further strengthen the role of eDNA in conservation and fisheries management(Kopp et al., 2023[55];).

Conclusions

While eDNA substantially broadens taxonomic coverage, conventional morphology remains indispensable and cannot be replaced at this stage because it provides vouchered specimens, diagnostic traits, and demographic information and verifies local occurrence. Using this combined approach, we detected 211 fish species spanning 17 orders, 71 families, and 146 genera across six representative rivers in Liaoning Province, whereas conventional surveys alone recorded 19 species from 4 orders, 9 families, and 19 genera. Cyprinidae dominated both datasets, and nine species recovered exclusively by conventional sampling underscore the continuing necessity of morphology-based methods.

Nevertheless, the results clearly demonstrate that eDNA greatly expands the range of taxa detected, improves the discovery of rare and concealed species and provides an efficient and scalable strategy for biodiversity monitoring at the river basin scale. Conventional methods remain essential for validating species presence and obtaining fundamental information on population attributes such as body size, age structure and health status. Therefore, integrating eDNA monitoring with targeted traditional sampling will enhance the comprehensiveness, accuracy and cost efficiency of fish diversity assessments in river ecosystems. This study provides practical guidance for applying eDNA-based monitoring in large river systems and supports scientific decision-making in freshwater conservation, fisheries resource management and ecological restoration in northeastern China and other temperate river basins.

Data availability

All data is stored in the GitHub repository https://github.com/SongShuang408/Comparison-between-eDNA-and-Traditional-Morphological-Methods.

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Declarations

Competing interests
The authors declare no competing interests.

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